The Antiproliferative Effects of Enzymatic Deglycosylation and Metabolic Undersulfation of Proteoglycans from the Cell Surface

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Enzymatic deglycosylation of plasma membrane proteoglycans and metabolic inhibition of glycosaminoglycan sulfation were employed as complementary methods to evaluate the effects of reduced cell surface content of functionally intact proteoglycans on the proliferative potential of cells. A Flavobacter heparinum extract, possessing multiple glycosaminoglycan substrate specificities, markedly inhibited the time-dependent expansion of BALB/c 3T3 fibroblast and human squamous cell carcinoma monolayers in culture and concurrently reduced the proportion of subconfluent cell populations in S-cell cycle phase by DNA flow-cytometry analysis. This antiproliferative effect was partially reproduced by lyases with heparan sulfate or chondroitin sulfate monospecificity, alone and in combination. The observed lability of heparan sulfate lyases I and II in serum-containing medium possibly hampered full reproduction of the effects of the multifunctional reagent. Growth inhibition of comparable magnitude was observed when glycosaminoglycan sulfation was metabolically blocked with sodium chlorate. The chlorate anion had its expected effect of substantially reducing sulfated glycosaminoglycan synthesis by the cells. Following release from serum deprivation, analysis of the progression of synchronized cell populations past the G1 restriction point suggested that in situ digestion with the glycosaminoglycan lyases limited, but did not delay, the numbers of cells entering S phase. These data support the hypothesis that plasma membrane proteoglycans mediate some of the cell-growth–promoting effects of serum factors via their glycosaminoglycan side chains. *J Invest Dermatol 97:43–49, 1991*

It is generally acknowledged that cellular proteoglycans, in their capacity as widely distributed and conserved plasma membrane constituents, participate in the organization of the extracellular matrix and in mediating the influences of matrix molecules and soluble ligands on cell growth and differentiation [1]. Reports of the copurification of proteoglycans and the actin cytoskeleton [2,3] provide for one potential mechanism by which transduction of the effects of matrix interaction on the cellular phenotype could occur. The identify of a proteoglycan core protein as one of the cell surface and matrix receptors for transforming growth factor-beta [4–6] further supports the role of the membrane-based proteoglycans as growth-regulating factors.

According to conventional understanding, the sulfated glycosaminoglycans (GAG) of cells function biologically in covalent linkage to core proteins of membrane-associated proteoglycans [1]. What the actual function of these complex carbohydrate side chains at the cell surface level might be, however, is less clear. Given the high negative charge of the GAG side chains, which suggests the potential for substantial biologic reactivity, it is reasonable to presume that they serve some of the ligand-binding functions of the proteoglycans. Increasing experimental attention is being directed at the precise molecular role of these heterogeneous polymers. Among other avenues of investigation, it has been shown that heparan sulfate proteoglycans in extracellular matrices sequester basic fibroblast growth factor and that the specific cytokine binding function is a property of the GAG side chains [7–9]. Thrombospondin, a matrix-associated glycoprotein that may function in cell proliferation, reportedly binds to the membrane-intercalated hybrid proteoglycan of mouse mammary epithelial cells via the heparan sulfate chains [10]. In the case of betaglycans, however, the heparan sulfate and/or chondroitin sulfate side chains are dispensable for binding of transforming growth factor-beta to the proteoglycan [11]. It has, furthermore, been asserted that in general the structure of the core protein is the principal determinant of the functional characteristics of a proteoglycan [12].

In consideration of the circumstantial evidence suggesting a growth-regulatory function for proteoglycan subsclasses, we present in this paper studies evaluating the role of the GAG side chains of heparan and chondroitin sulfate proteoglycans in the proliferation of BALB/c 3T3 fibroblasts and human squamous carcinoma cells. The experimental protocols employed two complementary methods to reduce the cell surface expression of functionally intact proteoglycans, namely, enzymatic deglycosylation with GAG-degrading lyases and metabolic inhibition of GAG polymer sulfation with sodium chlorate, in a test of the hypothesis that the GAG side chains are necessary for maximal serum-dependent cellular growth.

MATERIALS AND METHODS

Materials Chromatographic supplies were from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ) and Bio-Rad (Richmond,

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Abbreviations:
CS: chondroitin 4- and 6-sulfates
DS: dermatan sulfate
GAG: glycosaminoglycan
HS: heparan sulfate
SCL: squamous carcinoma line

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CA). Tissue culture procedures utilized flasks and 6-well plates from Corning Glassworks (Corning, NY), Eagle's modified minimum essential medium from Flow Laboratories (McLean, VA), and fetal bovine serum from Irvine Laboratories (Santa Ana, CA). GAG standards were heparan sulfate, prepared from Upjohn Co. (Kalamazoo, MI) bovine lung side fraction [13,14], and chondroitin 6-sulfate and hyaluronic acid, both purchased from Sigma (St. Louis, MO). $Na_2[^{35}S]O_4$ (370 mCi/mM), D-6-[^{3}H]-glucosamine HCl (30 Ci/mM), and [*Methyl*-^{3}H]-thymidine (20.0 Ci/mM) were products of Du Pont-New England Nuclear (Boston, MA). Sodium chlorate, testicular hyaluronidase, and chondroitinase ABC were obtained from Sigma. A semi-purified, multifunctional Flavobacter heparinum "heparinase," which is active against all GAG except for keratan sulfate and has negligible protease activity [15,16], and purified heparitinase I were prepared as described [17]. The multifunctional heparinase is comparable to that used in the report of Gill et al [15], which can be consulted for its activity against cell surface GAG during in situ digestion. Heparitinase I and II were also purchased from Sigma.

Cell Lines BALB/c 3T3 fibroblast clone A31 was obtained from the American Type Culture Collection (Rockville, MD). The human squamous cell carcinoma line (SCL), originally isolated by N. Fusenig (Heidelberg, FRG), was provided by G.G. Krueger (University of Utah). The lines were maintained in 25-cm^2 flasks, employing medium supplemented with 10% bovine serum and 80 μg/ml gentamycin sulfate.

Labeling of Cellular Glycosaminoglycans Cellular GAG were metabolically labeled, in duplicate cultures, under exponential growth conditions by 48-h incubations with [^{35}S]-sulfate (66 μCi/ml) and [^{3}H]-glucosamine (7 microCi/ml). After labeling, the medium fractions were decanted, and to each of these were added 2 mg of chondroitin 6-sulfate, as carrier, and cetylpyridinium chloride to a final concentration of 0.5%. The precipitates were washed with sodium chloride-saturated ethanol and taken up in 0.1% sodium dodecyl sulfate prior to analysis. Labeled products in association with the cell layers were extracted for 30 min at 37°C with 0.2% Triton X-100 in 25 mM Tris-HCl, pH 7.5, with 10 mM N-ethylmaleimide, 100 mM epsilon-aminocaproic acid, 10 mM EDTA, and 5 mM benzamidine HCl [2].

Analytical Methods The cell and medium preparations, obtained as above, were added to 110 × 1.0-cm columns of Sepharose CL-4B, using 0.1% sodium dodecyl sulfate with 0.35 M NaCl, 50 mM Tris-HCl, and the protease inhibitors, pH 7.5, as the eluant. The general analytic methods were as reported [18,19]. In brief, a combination of specific enzymatic digestion, with testicular hyaluronidase and chondroitinase ABC, and nitrous acid deaminative depolymerization at pH 1.0 [20] were used to identify specific GAG after their release from the proteoglycans by alkaline elimination. The digestion products were separated on Sephadex G-50 Superfine (50 × 0.4 cm), with 0.2 M NaCl in 10% ethanol as eluant. The uronic acid content [21] of the carrier GAG was used to follow the eluting substances.

Growth Assay For the assay of cell growth, the Millicell-HA culture disc system (Millipore, Bedford, MA) was adapted with the assistance of G.G. Krueger (University of Utah). The Millicell discs (4.2-cm^2 area covered with cellulose) were positioned in 6-well Corning plates. A silicone Millipore gasket and Teflon ring (10-mm diameter) were placed in the center of the Millicell, providing a confined area for cell inoculation at 10^5 cells/cm^2. After cell attachment for 24 h, the gasket and ring were removed, leaving circular cell monolayers. Medium was changed every 48 h, with or without added sterile GAG lyase or with sodium chlorate as a separate method (see below). At various times, duplicate Millicells were removed, washed with PBS, and fixed in 90% ethanol. The membranes were separated from the discs and stained with hematoxylin and eosin, and the areas of the 1:1 xerographic images of the growth patterns were quantified by planimetry (Sigma Scan, Jandel Scientific, Corte Madera, CA). En face and vertical sections through the membranes were examined histologically for morphologic effects of treatment.

In Situ Digestion of Cellular GAG with Lyases The general protocol involved the treatment of exponential cell cultures, either in Millicells or simply in 6-well culture plates, with the multifunctional heparinase or purified GAG lyases, alone and in combination. Our past studies have shown these enzymes to efficiently digest in situ the GAG associated with the external surfaces of a diversity of cell types, without appreciably affecting cell viability or histologic morphology [19,22, see also 15,23]. The multifuctional heparinase was used at concentrations of 0.1–1 mg/ml in complete growth medium with 10% calf serum beginning either 2 or 24 h after culture inoculation, depending on the experiment, and fresh enzyme was added anew with medium change every 2 d. Boiled heparinase and buffer alone were assayed in parallel as controls. In experiments involving the purified lyases, heparitinase I was used at 1 or 2 U/ml, heparitinase II at 4 U/ml, and chondroitinase ABC at 0.1–0.2 U/ml.

Sodium Chlorate Inhibition of GAG Sulfation Treatment of the cell cultures with sodium chlorate during the growth assays afforded an alternative method to in situ enzymatic digestion for assessing the growth consequences of interference with proteoglycan expression on cell surfaces. Chlorate inhibits sulfation of the GAG side chains, apparently without altering numbers or sizes of these polymers that are attached to the core proteins [24]. Two concentrations, 10 and 20 mM, were used during the exponential phases of cell growth, beginning 24 h after culture inoculation, and fresh chlorate was added with medium change every 2 d. Some culture wells were metabolically labeled for GAG content, as above, during treatment with chlorate as a control for its effects on the GAG synthetic phenotype. Parallel cultures to which had been added 20 mM NaCl in complete growth medium were used to control for the possible effects of increased osmolarity, as such, of chlorate on cell growth.

Thymidine Uptake and Cell Cycle Analysis DNA flow cytofluorometry of random cycling cell populations from 6-well Corning plates utilized propidium iodide staining of ethanol-fixed cells [25] under experimental and control conditions. Cells were inoculated into 6-well plates at 10^4 cells/well in complete medium and 10% serum. After 1 d in culture, synchronized populations for [^{3}H]-thymidine uptake were obtained by block at the G1 restriction point with medium containing 0.5% calf serum for 48 h. Progression through G1 commenced with the addition of 10% serum and 0.1 microCi/ml [^{3}H]-thymidine, with or without GAG-degrading enzymes. At intervals until past the first wave of DNA synthesis, wells were sampled for cell number and radioactivity quantified by liquid scintillation of the trichloroacetic acid precipitates solubilized with 0.2 N NaOH.

RESULTS

Cell Growth on Millicells The growth areas of the cell monolayers on the Millicell membranes were quantified by planimetry under control and experimental conditions, according to *Materials and Methods.* Figure 1 illustrates the measured growth areas of BALB/c 3T3 fibroblasts treated with the Flavobacter heparinum-derived multifunctional heparinase. The results are expressed as percent of the untreated duplicate controls sampled at the indicated time points. The heparinase reagent, which has activity against all GAG except for keratan sulfate, was added 24 h after culture inoculation and again with medium change every 2 d. The data show a dose-response effect over the concentration range that was employed (0.1–0.3 mg/ml in serum-containing medium), producing a growth area that was 43% of the control value at 13 d of growth for the highest concentration ($p < 0.025$, by the two-tailed t test). Loss of effect at the two lower concentrations (0.1 and 0.2 mg/ml) after 10 d in culture likely reflects insufficient enzyme for substrate.

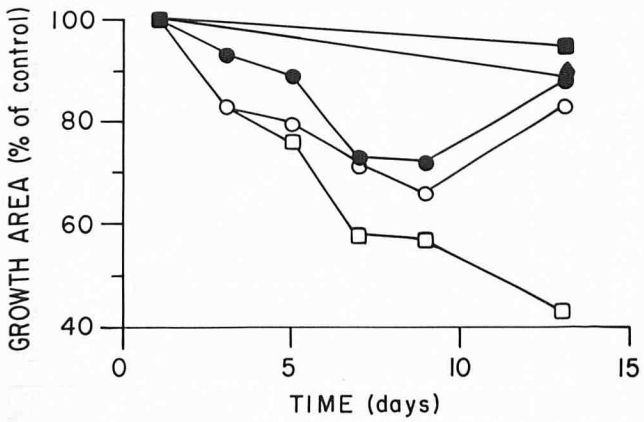

Figure 1. Growth areas of BALB/c 3T3 fibroblasts on Millicells in presence of multifunctional heparinase. After 24 h growth on Millicells, the multifunctional heparinase was added at three concentrations to a series of duplicate wells. Untreated cultures and those treated with boiled heparinase or phosphate-buffered saline alone comprised the controls. At the indicated time points in culture, wells were harvested and the growth areas quantified and expressed as percent of the growth areas of concurrently harvested control cultures, i.e., those without added buffer or enzyme (*solid squares,* buffer control; *solid triangles,* boiled heparinase; *solid circles, open circles,* and *open squares,* heparinase at 0.1, 0.2, and 0.3 mg/ml, respectively).

Boiled heparinase (0.3 mg/ml) and phosphate-buffered saline (to control for dilution) were the negative controls. Determination of 3T3 cell numbers in the presence of the multifunctional heparinase, boiled heparinase, or buffer alone (Fig 2) paralleled the parameter of growth area. The treatments had no discernible cytolytic effects, nor were there alterations in cell morphology by microscopic examination of the ethanol-fixed and hematoxylin and eosin-stained monolayers attached to the Millicell membranes. Cytolic effects were controlled by Trypan blue dye exclusion of cultures grown in the presence or absence of 0.3 mg/ml heparinase. Following detachment by brief trypsin/EDTA treatment, the percentages of cells from each culture that were viable by this criterion were 86% versus

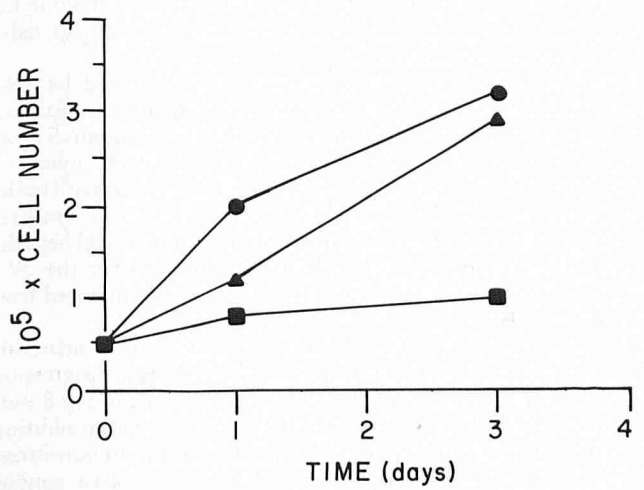

Figure 2. Multifunctional heparinase effect on BALB/c 3T3 cell growth. The heparinase was added 2 h after culture inoculation. Over a 3-d period of growth in culture wells, cell numbers were quantified in wells treated with multifunctional heparinase (1 mg/ml, *solid squares*), boiled heparinase (1 mg/ml, *solid triangles*), and phosphate-buffered saline *(solid circles)*.

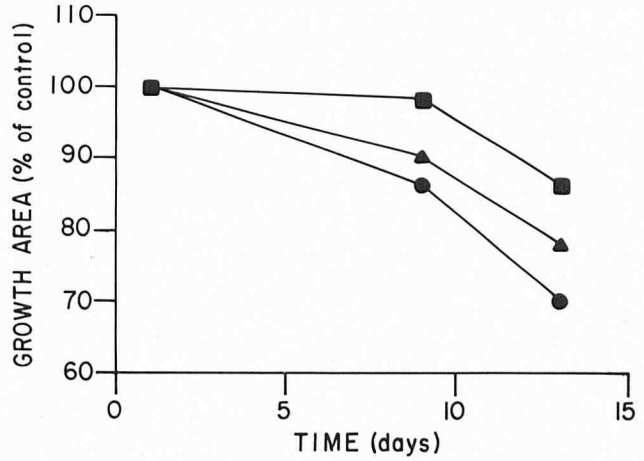

Figure 3. Growth areas of BALB/c 3T3 fibroblasts on Millicells in presence of purified lyases. With the Millicell growth area assay, the effects of chondroitinase ABC (*circles,* 0.2 U/ml; *triangles,* 0.1 U/ml) and heparitinase I (*squares,* 2 U/ml) on cell growth were quantified. Results are expressed as percent of concurrently sampled, untreated control cultures.

90% at day 3 of culture, 82% versus 80% at day 7, and 90% versus 92% at day 13, for the heparinase versus control cultures respectively.

To establish that the preceding results represented effects on cell proliferation, as such, rather than cell migration, newly inoculated cultures on the Millicells were sublethally irradiated with 1500 Rads of cesium (Gammator, Isomedix Corporation). Following irradiation, both heparinase-treated and control cultures failed to expand their growth areas, compared with nonirradiated cultures (not shown), suggesting that the measured parameter of growth area is largely dependent on proliferation.

Purified lyases were substituted in the Millicell assays to substantiate the observed antiproliferative effects of the multifunctional heparinase. With BALB/c 3T3 (Fig 3) and SCL (Fig 4) cells, chondroitinase ABC and heparitinase I partly reproduced the results obtained with the multifunctional heparinase. At day 13, chondroitinase ABC (0.2 U/ml) reduced the growth area to 70% and 65% of controls for 3T3 and SCL cells, respectively, and heparitinase I reduced growth in both cell lines to ~85% of the control. Fifty percent growth inhibition of the SCL cells was evident at 13 d of

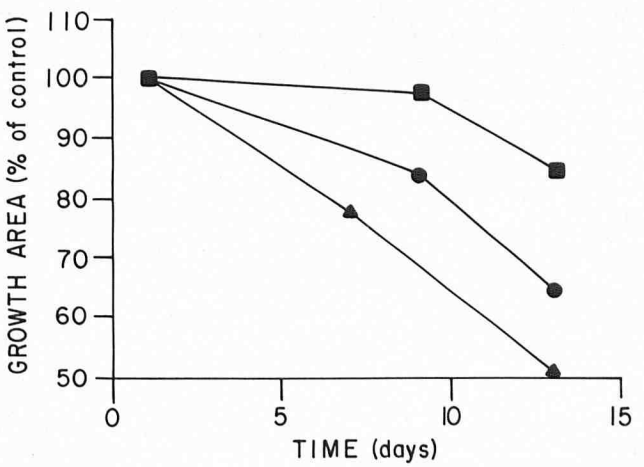

Figure 4. Growth areas of SCL cells in presence of purified lyases. As in the experiment depicted in *Fig 3,* SCL cells were cultured on Millicells in the presence of heparitinase I (*squares,* 2 U/ml), chondroitinase ABC (*circles,* 0.2 U/ml), and multifunctional heparinase (*triangles,* 0.3 mg/ml).

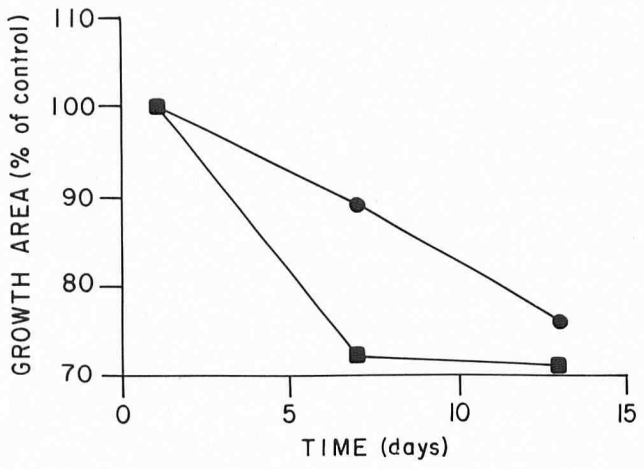

Figure 5. Sodium chlorate effect on growth areas of BALB/c 3T3 fibroblasts on Millicells. In this experiment using the Millicell assay, the cells were treated with either 10 mM *(circles)* or 20 mM sodium chlorate *(squares)* during the observed period of growth. The resultant growth areas were referenced against those of the untreated control cultures. *Data points,* means of quadruplicate cultures.

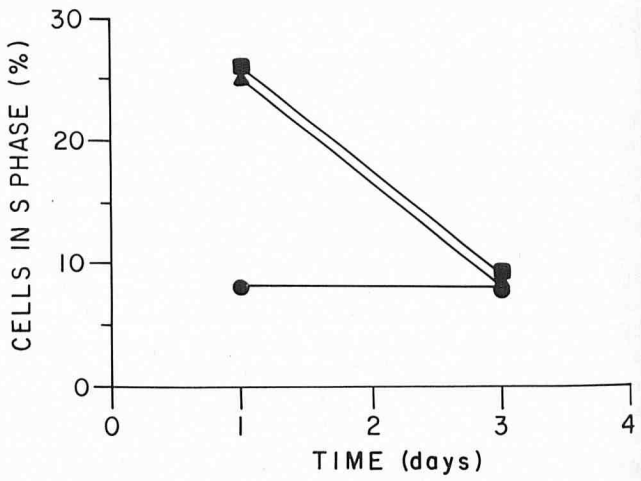

Figure 6. DNA flow cytofluorometry of BALB/c 3T3 cell populations treated with multifunctional heparinase. Cultures in 6-well plates were treated with phosphate-buffered saline *(squares),* multifunctional heparinase (*circles,* 1 mg/ml), or boiled heparinase (*triangles,* 1 mg/ml), beginning 2 h after cell inoculation. At 1 and 3 d of growth, the cultures were harvested and the cell population cycle distributions were determined by DNA flow cytofluorometry. Percent of each cell population in S phase is depicted.

culture with the multifunctional heparinase. The incomplete duplication of the latter's effects by the purified lyases may have been due to suboptimal concentrations or, in the case of heparitinase I, lability in the serum-containing medium (see below).

Chlorate Effects on Cell Growth The use of sodium chlorate afforded an additional method to assess the growth effects of reduced cellular content of functional proteoglycans [24]. Two concentrations, 10 and 20 mM, were used in the medium of BALB/c 3T3 cell cultures and were added again with each medium change. At 1, 7, and 13 d of growth on the Millicell discs, duplicate wells were harvested and the growth areas quantified by planimetry. The results depicted in Fig 5, for which the data have been referenced against untreated control-culture growth areas, indicate growth inhibition in a dose- and time-dependent fashion. Approximately 30% growth inhibition was observed with 20 mM chlorate at 13 d of culture. As with the GAG lyase studies, there were no evident morphologic effects on the cells by chlorate treatment. Control cultures grown in medium supplemented with 20 mM NaCl had growth areas that were not distinguishable from those of the other controls.

To establish that chlorate had its intended effect of inhibiting synthesis of sulfated GAG, cultures so treated were concurrently labeled with [^{35}S]-sulfate for 48 h, and the total GAG synthesized during the labeling period were analyzed (Table I). Quantities of sulfated GAG, based on [^{35}S]-sulfate incorporation, were reduced to ~30–40% of control levels under the two chlorate concentrations (10 and 20 mM).

Table I. Chlorate Effects on Glycosaminoglycan Synthesis[a]

	10^{-3} × [^{35}S]-Sulfate DPM[b]		
	Product		
Condition	HS	CS	DS
Control	100/35%	90/32%	95/33%
Chlorate (10 mM)	40/39%	26/25%	37/36%
Chlorate (20 mM)	32/38%	28/33%	25/29%

[a] BALB/c 3T3 Fibroblasts were labeled for 2 d with [^{35}S]-sulfate in the presence (10 and 20 mM) or absence of sodium chlorate. The total amounts of GAG synthesized and the class distribution were determined according to *Materials and Methods.*

[b] Data are expressed as both dpm × 10^{-3} and percentage distribution of product under each condition for the 3 product classes. HS, heparan sulfate; CS, chondroitin 4- and 6-sulfates; DS, dermatan sulfate.

Cell Cycle Effects of Lyase Digestion Random cycling 3T3 and SCL cells in the presence or absence of multifunctional heparinase (1 mg/ml) were examined by DNA flow cytofluorometry. Boiled heparinase and phosphate-buffered saline served as controls. After 1 d in culture, 3T3 cells treated with either buffer or boiled heparinase were in exponential growth, with ~25% of cells in S phase (Fig 6). In contrast, the cells in multifunctional heparinase, whereas morphologically similar to controls, were relatively quiescent (5–10% in S phase). After 3 d in culture, the control cells were confluent, with <10% of the cells now in S phase. At this time, the actively treated cells were nonconfluent, yet the S-phase DNA content was low. Thus, heparinase treatment markedly inhibited the cell-cycle competence of newly plated cells. Results with the SCL line were the same. In this assay, 4 U/ml heparitinase II alone had no discernible effects, in comparison with untreated controls, using the 3T3 cultures (not shown).

Using 3T3 and SCL cell populations synchronized by 48-h growth in 0.5% serum, the effect of multifunctional heparinase treatment on progression past the G1 restriction point into S phase was examined. During continuous labeling with [^{3}H]-thymidine after addition of 10% serum (Fig 7), control cells treated with boiled heparinase entered S phase at ~15 h, with maximal DNA synthesis at ~20 h. Cells treated continuously with multifunctional heparinase, however, showed baseline thymidine uptake over the 37-h course of the experiment and were thus markedly inhibited from entry into S phase.

A combination of purified lyases was used to substantiate the effects of the multifunctional heparinase on cell-cycle progression. In the absence of GAG-degrading enzymes, the data in Fig 8 indicate entry of 3T3 cells into S phase ~12–13 h after serum addition. Cells treated continuously with a combination of chondroitinase ABC, heparitinase I, and heparitinase II entered S phase concurrently with controls, but at reduced numbers. Differences between means are significant at $p < 0.05$ for the 21-, 23-, and 37-h time points (two tailed t test).

Lability of Heparan Sulfate Lyases in Medium The purified lyases incompletely duplicate the inhibitory effect of the multifunc-

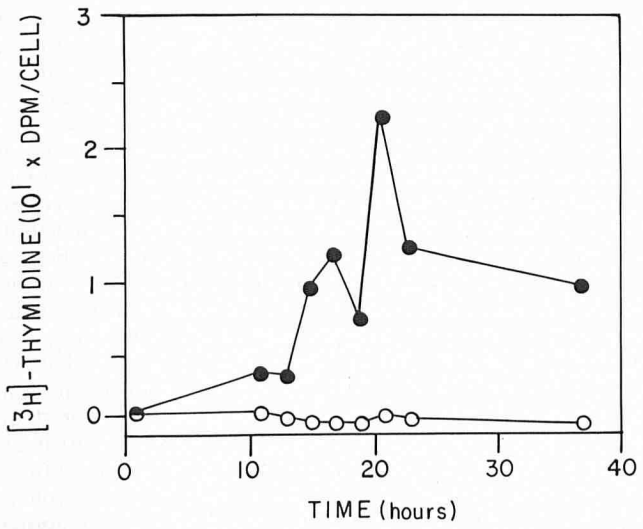

Figure 7. Effect of multifunctional heparinase on entry of a synchronized cell population into S phase. BALB/c 3T3 and SCL cells were released from block at the G1 restriction point with medium containing 10% serum, 0.1 microCi/ml [^{3}H]-thymidine, and either heparinase or boiled heparinase. At the indicated times thereafter, BALB/c 3T3 cultures (SCL cultures gave similar results) treated with 1 mg/ml multifunctional heparinase *(open circles)* or boiled heparinase *(solid circles)* were sampled and [^{3}H]-thymidine uptake into DNA quantified in reference to cell counts.

tional heparinase on cell growth. One explanation for this could be lability of the heparitinases in serum-containing medium. This possibility was assessed by analyzing heparitinase activity on an authentic standard heparan sulfate substrate under the conditions of cell culture that were utilized. Heparitinase I (2 U/ml) and heparitinase II (4 U/ml) were added individually to 3T3 cell cultures in wells

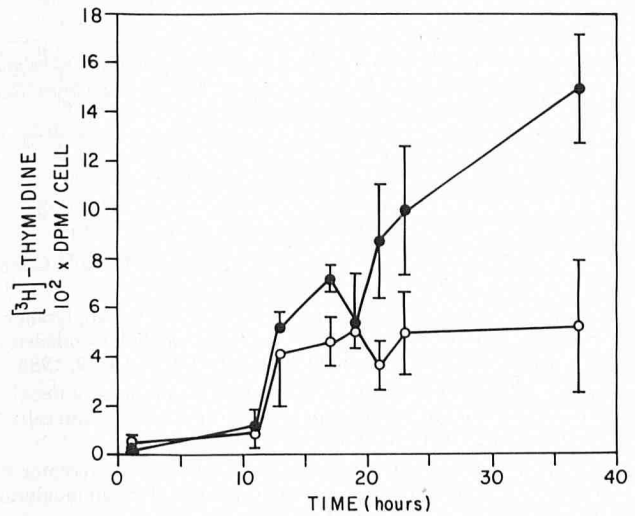

Figure 8. Effect of lyase combination on entry of a synchronized cell population into S phase. As in the experiment depicted in *Fig 7*, the entrance of synchronized BALB/c 3T3 fibroblast populations into S phase was analyzed in the presence *(open circles)* or absence *(solid circles)* of a combination of chondroitinase ABC (0.1 U/ml), heparitinase I (1 U/ml), and heparitinase II (4 U/ml). The data are expressed as means ± SD of replicate control and treated cultures at each time point.

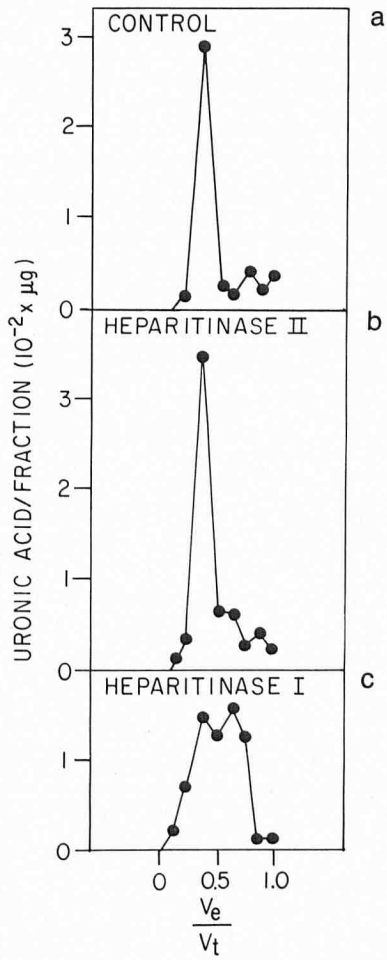

Figure 9. Lability of heparan sulfate lyases in serum-containing medium. *(a)* Sephadex G-50 elution pattern, by uronic acid analysis, of heparan sulfate from control cell culture supernatants in the absence of GAG lyases. *(b)* Heparan sulfate elution profile from cultures containing heparitinase II. *(c)* Elution from cultures with heparitinase I.

containing medium with 10% serum and 1 mg of bovine lung heparan sulfate. Twenty-four hours after enzyme addition, the media samples were collected and chromatographed on Sephadex G-50 (Fig 9). Compared to the negative control cultures without enzyme (Fig 9*a*), heparitinase II proved largely inactive on the heparan sulfate substrate (Fig 9*b*). In contrast, somewhat more than one-half of the substrate was digested to oligosaccharide products by heparitinase I (Fig 9*c*). In buffer alone, 1 mg/ml heparan sulfate is completely degraded at these enzyme concentrations.

DISCUSSION

The current studies were founded on the premise that GAG side chains of the plasma membrane proteoglycans serve as specific receptors for some of the growth-promoting factors in serum. We tested the hypothesis that expression of fully glycosylated and sulfated proteoglycans is necessary for maximal cell proliferation. The two experimental approaches were enzymatic deglycosylation with GAG-degrading enzymes and metabolic inhibition of GAG sulfation with sodium chlorate. Cell growth was assessed in several ways, including proliferation-dependent expansion of cell monolayers on Millicell membranes, DNA flow cytometry of randomly cycling

cell populations, and the capacity of synchronized cells to progress past the G1 restriction point into S phase. Initial enzymatic studies employed a Flavobacter heparinum extract, with multiple GAG substrate specificities and negligible protease activity [15,16], which efficiently removes > 90% of the GAG side chains of proteoglycans from cell surfaces without appreciably affecting cell morphology or viability ([19,22]; see also [15]). In the growth assays this multifunctional heparinase substantially inhibited, in a concentration-dependent fashion, the growth of BALB/c 3T3 fibroblasts and squamous carcinoma cells (Figs 1 and 2) by blocking the capacity of subconfluent cells to transit the cell cycle beyond the G1 restriction point (Figs 6 and 7). Monospecific GAG lyases, alone and in combination, only partly reproduced the effects of the multifunctional heparinase reagent (Figs 3, 4, and 8). With a separate method, however, cell growth was significantly inhibited in the presence of sodium chlorate (Fig 5), concurrent with reduction in sulfated GAG synthesis (Table I). Collectively, therefore, the data support the proposal that GAG side chains of the proteoglycans function as permissive factors for serum-dependent cell growth in vitro.

Among the explanations for the failure of the purified lyases to completely reproduce the multifunctional heparinase effects, one possibility is related to the observation that the heparitinases, especially II and to a lesser extent I, are somewhat unstable in serum-containing medium (Fig 9). Another consideration is that the multifunctional heparinase contains numerous enzymes covering a broad range in substrate specificities (including disaccharidases) that are not represented in the simple combinations that were tested. The full effects may require the concerted action of multiple enzymes. Indeed, Gill et al [15] showed that the in situ digestion of cellular GAG achieved with the multifunctional heparinase leaves only small "stubs" attached to the core proteins, an efficiency that is only partially reproduced by a combination of heparitinase I and chondroitinase ABC. Limited availability of fully active heparan sulfate lyases is a factor that hinders optimally high concentrations of these purified reagents in the growth assays. Whereas it is admittedly possible that Flavobacterial heparinase contains a heat labile factor that inhibits cell growth but is not a GAG-degrading enzyme, the chlorate data, discussed below, would suggest otherwise. The present studies, however, do not completely distinguish between the two possible interpretations.

Sulfation of GAG is efficiently suppressed by chlorate through its potent competitive inhibitory effect on sulfate adenylyltransferase, giving rise to a reduction in the synthesis of the sulfate donor, adenosine 3'-phosphoadenylylphosphosulfate [24,26,27]. Chlorate also inhibits sulfation of tyrosyl residues of proteins, without inhibition of protein synthesis or toxic effects on cells [28]. In studies of GAG biosynthesis, chlorate has been shown to affect the sulfation patterns of these carbohydrates, but not the numbers or sizes of the polymers comprising the complete proteoglycans [24,27]. Sulfation of chondroitin sulfate is more affected than that of heparan sulfate [24,26,27]. Epimerization of D-glucuronic to L-iduronic acid is also reduced by chlorate [24,27].

Under the conditions of growth assay used in the present studies, 10 and 20 mM chlorate had a clear inhibitory effect on cell growth, resulting in a 30% decrease in growth area compared to the controls at 13 d of culture (Fig 5). In contrast, Keller et al [24] stated that there were no changes in the growth of Swiss mouse 3T3 cells in 5 mM chlorate, despite alteration in the morphologic appearance of the cells. A subtle growth effect of 1 and 5 mM chlorate, however, is suggested by the growth curves presented in Fig 1 of their report, an effect interpreted to be a decrease in the saturation density of the cells [24]. It seems plausible that higher chlorate concentrations or perhaps a more quantitative assay for growth, such as the Millicell system described herein, would have allowed a growth-inhibitory effect to become more evident. The inhibition of cell growth by chlorate observed in our studies must be evaluated in light of the possibility of pleiotropic effects on cellular metabolic pathways other than those of the proteoglycans. Given the complementary data from GAG lyase digestion of cell cultures, it is our opinion that with the substantially reduced GAG side chain sulfation produced by chlorate, the ligand binding functions of the proteoglycans are likely to be perturbed, and the overall functional effect on cell growth could be similar to that of enzymatic deglycosylation.

The precise functions of pericellular proteoglycans have been remarkably elusive. Even more obscure has been the physiologic significance of the GAG side chains. Whereas heparan sulfates have long been considered to mediate certain adhesive response to matrices (e.g., [29]), it has proved difficult to establish that metabolic inhibition of GAG sulfation [24], enzymatic digestion of heparan sulfate from cells [23,30,31], or mutations in the GAG synthetic pathway [32] materially affect cellular adhesion to artificial substrates or matrix molecules in vitro. Similarly, the novel suggestion that the core protein of a skin fibroblast proteoheparan sulfate is a physiologic transferrin receptor, which can be modified in its affinity for the ligand by the presence or absence of the GAG side chains [33], has apparently not been verified [34]. Considerable early circumstantial evidence on transformation-associated changes in the GAG synthetic phenotype (e.g., [35]) had implied a central role for these polymers in the growth regulation of cells. The observation of heparan sulfate chains in the nuclei of hepatocytes under certain growth conditions [36], the reduced tumorigenicity of Chinese hamster ovary cell mutants defective in heparan sulfate proteoglycan synthesis [37], and the effects of growth and differentiation on proteoglycan and GAG free-chain expression in cultured human epidermal keratinocytes [38–40] have led to similar conclusions. There has not emerged, however, unequivocal evidence for their direct and etiologic function in growth control.

Recent experimental attention has been directed at the functional interaction of proteoglycans with growth factors. Subspecies of heparan sulfate proteoglycans from endothelial cells, for example, modulate in different directions the mitogenicity of acidic fibroblast growth factor on this cell type, and the biologic activity resides in the GAG side chains [41]. The results of the present cell growth, cytofluorometry, and thymidine uptake studies indicate that enzymatic removal of GAG side chains from cell surface proteoglycans, or inhibition of their sulfation, partly suppresses cell proliferation, apparently by influencing the competence of cells to transit the cell cycle beyond the G1 restriction point. A reasonable hypothesis, in general accord with these experimental observations and with the relevant literature, is interference with the interaction of as-yet unspecified growth-promoting ligands and their GAG receptors.

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